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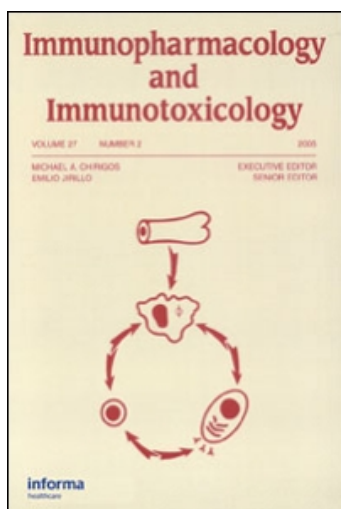
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Silica Induced Suppression of the Production of Third and Fifth Components of the Complement System by Human Lung Cells In Vitro

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SILICA INDUCED SUPPRESSION OF THE PRODUCTION OF THIRD AND
FIFTH COMPONENTS OF THE COMPLEMENT SYSTEM BY HUMAN
LUNG CELLS *IN VITRO*¹

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ABSTRACT

Although investigations to date have demonstrated the ability of the monocyte/macrophage to synthesize complement components, only a limited number of studies on complement synthesis by nonhepatic tissue cells have been reported. To begin to fill this gap in our knowledge we have recently evaluated the ability of lung tissue cells to synthesize and secrete various complement components *in vitro*. Using ³⁵S-methionine incorporation and immunoprecipitation techniques we have previously demonstrated the ability human lung type II pneumocytes (A549) and human lung fibroblasts (WI-38), to synthesize and secrete a variety of both early and terminal complement components, as well as several regulatory proteins including C1r, C1s, C4, C3, C5, C6, C7, C8, C9, Factor B, Factor H, Factor I and C1s inactivator. Our present studies demonstrate the capability of silica to regulate complement component production by A549 cells, but not complement component production by WI-38 cells. Specifically, using sensitive ELISAs we demonstrated that a non-toxic dose of silica had the capability to suppress the production of both C3 and C5 by A549 pneumocytes by 40-50 percent, but had no effect on C3 or C5 synthesis by WI-38 fibroblasts. Additionally, using ³⁵S-methionine incorporation and TCA precipitation techniques, we demonstrated that suppression of C3 and C5 production by silica treated A549 pneumocytes was not a result of suppression of total protein synthesis. These studies demonstrate that silica, which has been implicated in pulmonary diseases, has the capability to regulate local complement production by lung tissue cells *in vitro*. *In vivo*, this suppression of complement production by

the type II pneumocytes could alter the local tissue reservoir of complement components during infection and pulmonary injury, thus resulting in depressed pulmonary host defense.

INTRODUCTION

Our understanding of the importance of the complement system in host defense and inflammation has grown significantly from the original observations over a century ago that serum contained heat labile proteins which were bactericidal⁽¹⁾. Today, the complement system is seen as a complex cascade of biochemical reactions which not only play a central role in host defense and inflammation, but have been implicated as regulatory molecules in immune responses as well as the coagulation pathway⁽²⁾. Previously, control of the tissue levels of individual complement components was generally believed to be a result of nonspecific diffusion associated with changes in vasopermeability. Thus, tissue complement concentrations were thought to be only a reflection of the humoral complement levels. More recently, questions have been raised about the existence and significance of local synthesis and secretion of complement components on the regulation of host defense and inflammation within tissues. Generally, the investigations into the question of cellular synthesis and secretion of complement components have focussed on the monocyte/macrophage. These investigations have demonstrated that the monocyte/macrophage has the capability to synthesize many complement components and regulatory proteins, including C1, C4, C2, C3, C5, Factor B, Factor D, Factor H, Factor I, properdin and C1 inhibitor (3,4,5).

Recent studies have reported that a variety of factors or agents can regulate complement production by the monocyte/macrophage *in vitro*. These agents include antigen sensitized sheep red blood cells, immune complexes, lipopolysaccharide and interferon-gamma (5,7,8,9). Although these studies have provided important insights into the potential contribution of recruited cells to local concentrations of complement components and regulatory proteins, very little is known about the contribution of resident or tissue cells to local complement levels. To begin to fill this important gap area, our laboratory has recently demonstrated the ability of prototypic lung cells, i.e. human type II pneumocytes (A549) and human lung fibroblasts (WI-38), to synthesize and secrete a variety of complement components *in vitro*, including C1s, C4, C3, C5, C6, C8 and C9 (10). In our

present studies we demonstrated the ability of silica to suppress complement component production by type II pneumocytes, but not WI-38 fibroblasts. Specifically, these studies demonstrated that although silica particles suppressed C3 and C5 protein production by A549 pneumocytes, silica had no effect on A549 cell viability or total protein synthesis by these cells, suggesting that suppression of both C3 and C5 production was a specific response rather than a generalized response of the type II pneumocytes to silica. Interestingly, the C3 and C5 levels produced by A549 pneumocytes cultured under optimal conditions, i.e. in the presence of FBS and absence of silica particles, were significantly higher than the C3 and C5 levels produced by A549 cells cultured in 1) the presence of FBS and silica, or 2) cells cultured in serum free conditions in the presence or absence of silica. These results suggest that complement production by A549 pneumocytes is responsive to not only silica, but also to culture conditions, i.e., influence of serum factors and/or nutrients. Thus, these data demonstrate that silica can suppress production of at least the third and fifth complement components by type II pneumocytes *in vitro*, and *in vivo* this suppression may have a major impact on not only host defense but also on the course of pulmonary inflammation by lowering tissue complement levels.

METHODS

A549 and WI-38 Cell Cultures

Passage 76 human lung type II pneumocyte cell line (A549, ATCC CCL 185), and passage 13 human lung fibroblast cell line (WI-38, ATCC CCL 75) were purchased from American Type Culture Collection (Rockville, MD). The A549 cell line was maintained in F12 Ham's medium (Kansas City Biological, Kansas City, MO) and the WI-38 cell line was maintained in Eagle's minimum essential medium (MEM) (Kansas City Biological, Kansas City, MO). Both types of media were supplemented with 15% fetal bovine serum (FBS) (Sterile Systems, Inc., Logan, UT), 20 µg/ml gentamicin, 0.5 µg/ml amphotericin B, 0.1 mM nonessential amino acids, 4 mM glutamine, and 1 ml/100 ml medium of MEM vitamins (100X) (Gibco, Grand Island, NY). Routine subcultures for A549 pneumocytes (passage 76-94) and WI-38 fibroblasts (passage 13-22) were done at 1:3 split ratios by

incubation with 0.05% trypsin 0.02% ethylene diaminetetraacetic acid in calcium-magnesium free Hank's Balanced Salt Solution (Gibco, Grand Island, NY) for 5-9 minutes at 37°C. Cell cultures were incubated at 37°C in a humidified incubator with 5% CO₂ in air.

Cell Culture Conditions for Sample Collection

The A549 and WI-38 cell lines were grown in 75cm² tissue culture flasks (Falcon, Oxnard, CA) in the appropriate complete media described above. Confluent WI-38 fibroblast cultures (1×10^7 cells/flask) or confluent A549 pneumocyte cultures (1×10^7 cells/flask) were used for all studies. Complement production experiments were begun by rinsing the confluent cell cultures 3 times with 10 ml sterile saline, followed by the addition of 10 ml of serum free or 15% FBS supplemented media with or without silica particles. These flasks were then incubated at 37°C in a humidified incubator with 5% CO₂ in air for 11 days. On day 1, 3, 5, 7 and 9, a 5 ml sample of medium from each cell culture flask was collected and replaced with 5 ml of the appropriate fresh medium. On the last day of culture (i.e. day 11), the total cell culture supernatant was obtained. All culture supernatant samples were centrifuged at 400 xg for 10 minutes to remove cellular debris then divided into 1 ml aliquots and stored at - 70°C.

Cell Culture with Silica Particles

Experiments examining the effects of silica particles on complement production by lung cells were initiated by adding a non-lethal dose of silica particles (1 mg/ 10^7 cells/flask) to confluent cell cultures. Silica particles were purchased from Sigma (Sigma Chemical Co., St. Louis, MO), and the particle size distribution was 0.5 to 10 microns with approximately 80% between 1 to 5 microns. Silica particles were cleaned by boiling a 3% (w/v) slurry in 1N HCl to remove contaminants such as Fe₂O₃, according to Dauber et al (11), then autoclaved before use.

Antiserum

The IgG fraction of goat anti-human C3 was purchased from Cappel Laboratories (Malvern, PA). Rabbit anti-human C5 antibody was prepared by immunizing rabbits subcutaneously with 250 µg purified human C5 (Calbiochem Biochemicals, San Diego, CA) in Freund's complete adjuvant (Difco Laboratories, Detroit, MI) (12). Serum was isolated from rabbit blood and tested for anti-human C5 antibody titer by double immunodiffusion analysis. Both normal human serum and C5 deficient human serum (13) were used to validate the specificity of the anti-human C5 antiserum. Rabbit anti-human C5 IgG was purified using a Protein A Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and assayed for purity by immunoelectrophoresis against purified C5, normal human serum, or C5 deficient human serum. Alkaline phosphatase conjugated to antiserum or antigen was prepared by the method of Voller et al (14). The enzyme preparation used was alkaline phosphatase, Sigma type VII-S (Sigma Chemical Co., St. Louis, MO) of specific activity 1000 units/mg.

ELISA for Human C3 Antigen

The immunoassay to quantitate human C3 antigen was performed in 96 well flat bottom microtiter plates (Flow Laboratories, McLean, VA) using a competitive enzyme-linked immunospecific assay (ELISA) technique (9,15). The lower limit of detection for this ELISA was 50 ng C3/ml. Because the A549 pneumocyte and WI-38 fibroblast cells were grown in media containing 15% FBS, the cross reactivity of the FBS was tested in this ELISA and was found to have no detectable cross reactivity.

ELISA for Human C5 Antigen

The immunoassay to quantitate C5 antigen was performed in 96 well flat bottom microtiter plates (Flow Laboratories, McLean, VA) using a competitive ELISA procedure (9,15). The lower limit of detection for this ELISA was 50ng

C5/ml. Because the A549 pneumocyte and WI-38 fibroblast cells were grown in media containing 15% FBS, the cross reactivity of the FBS in this ELISA was tested and no significant cross reactivity was found.

Functional Assay for Human C5

A hemolytic assay to quantitate functional C5 (C5 H50) was performed using antibody-coated sheep erythrocytes in veronal buffered saline with 0.1% gelatin, 0.15mM Ca^{++} , and 1.0mM mg^{++} . Generally, 250 μl of purified C5 (Calbiochem Biochemicals, San Diego, CA) or test sample was added to 250 μl of a 4.0% solution of C5 deficient serum (14). Next, 125 μl of antibody-coated sheep erythrocytes (3.33×10^7 cells/ml) was added to the mixture, followed by a 60 minute incubation at 37°C. Following incubation, 1.0 ml isotonic saline was added and the samples were centrifuged at 450 x g for 6 minutes. Hemolysis was determined by evaluation of the resulting supernatants at the optical density of 412 nm. Results were calculated by comparing the dilution at which 50% red blood cell lysis occurred in relation to the highly purified C5 which was used as the standard in each assay. The 50% lysis point for this assay was approximately 1-5 ng C5/ml, and no cross reactivity between human C5 and FBS was detected using this functional assay.

Silica and A549 Pneumocyte Culture Supernatants

To eliminate the possibility that depressed levels of antigenic C3 seen in the experiments described in the Results Section were simply a result of non-specific sticking of C3 molecules to silica particles, studies were done to determine the direct effect of silica on C3 levels in culture supernatants obtained from A549 pneumocytes. For these studies 1 mg of silica was incubated with 10 mls of A549 pneumocyte culture supernatant for 30 minutes and the resulting antigenic C3 levels were determined by ELISA for C3 antigen. These studies demonstrated that antigenic C3 levels in silica treated and nonsilica treated culture supernatants were identical (data not shown).

Protein Synthesis by A549 Pneumocytes

To evaluate total protein synthesis, incorporation of radioactive amino acids into proteins synthesized by A549 pneumocytes was utilized. For these studies the cells were grown to confluence in 48 well plates and maintained in serum free, or serum containing media methionine free F12 Ham's medium for 3 days in the presence of 50 $\mu\text{Ci/ml}$ L-[^{35}S] Methionine (1120 $\mu\text{Ci/mmol}$, New England Nuclear, Boston, MA). At selected times in culture, after exposure to silica particles, cell supernatants were collected, centrifuged 400 xg for 10 minutes, aliquoted and stored at -70°C . These culture supernatants were utilized for analysis of total protein synthesis using TCA precipitation, i.e., equal volumes of ^{35}S -methionine labelled cell culture supernatant and 10% TCA, and 1% albumin as carrier protein, were incubated at 4°C for 30 minutes and then centrifuged at 400 xg for 20 minutes to precipitate ^{35}S -methionine incorporated proteins. These same ^{35}S -methionine labelled A549 pneumocyte culture supernatants were examined for qualitative differences in total protein synthesis and secretion after exposure to silica particles by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (16). After electrophoresis the SDS-PAGE gels were fixed, vacuum dried, and radiolabelled proteins were visualized by autoradiography.

Protein Assay

A549 pneumocyte and WI-38 fibroblast serum free culture supernatants were analyzed for total protein content using a microprotein assay with a sensitivity range of 1 to 10 μg protein/0.1 ml as described by Bradford (17).

Cell Viability

Cell viability was examined by uptake of vital dye Erythrocin B (J.T. Baker Chemical Co., Phillipsburg, NJ) or Trypan Blue (Gibco Laboratories, Grand Island, NY) and visualized by light microscopy. In addition, lactate dehydrogenase (LDH) levels in cell culture supernatants were also used as an indicator of cell death

or cell integrity (18). All LDH data was expressed as the percent of total LDH, where total LDH equaled the LDH released into the supernatant from 1×10^7 A549 pneumocytes after exposure to Triton X-100. The percent LDH was calculated using the following equation:

$$\% \text{ LDH} = \frac{\text{LDH content in experimental culture supernatant}}{\text{Total LDH}} \times 100$$

Morphologic Evaluation of Silica Effects on A549 Pneumocytes

Light Microscopy

Interactions of silica particles with adherent (cultured) and nonadherent (suspension) A549 pneumocytes were evaluated by light microscopy. A549 pneumocytes were cultured as described above for 5 days in the presence or absence of silica particles ($1 \text{ mg}/10^7$ cells/flask). On day 5 adherent A549 pneumocytes were removed from the tissue culture flasks by trypsinization, then washed 3x and resuspended in F12 Ham's medium. Wet mounts were made of A549 cells that had been cultured in the presence or absence of silica particles and evaluated by light microscopy. To eliminate the possibility that interactions of silica particles and adherent A549 pneumocytes were artifacts of sample preparation for microscopy, adherent A549 pneumocytes cultured for 5 days in the absence of silica particles were trypsinized, washed, and resuspended in F12 Ham's medium containing silica particles ($1 \text{ mg}/10^7$ cells/10 ml). These nonadherent A549 pneumocyte suspensions were incubated at 37°C for 30 minutes, then wet mounts were made and evaluated by the light microscopy.

Electron Microscopy

To evaluate the ultrastructural effects of silica on A549 pneumocytes, individual A549 cell cultures were drained of culture fluid and rinsed quickly with 0.2M cacodylate buffer. The plate was flooded with 4% cacodylate buffered glutaraldehyde and allowed to fix for one hour at room temperature. It was then

post-fixed with 2% osmium tetroxide for 30 minutes. The plates were rinsed with buffer and the cells scraped with a rubber policeman. The cells were collected in 1.5 ml centrifuge tubes and centrifuged in a Beckman Microfuge B. The resulting pellet was dehydrated through a graded series of alcohol, propylene oxide, and embedded in an Epon-Araldite mixture. One micron thick sections were cut and stained with toluidine blue and several blocks were selected for thin sectioning (60 mμ). Thin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken on a Philips EM 300.

Data Analysis

Quantitation of absolute complement component levels in individual cell culture supernatants were determined by direct comparison to the standard curves of known C3 and C5 antigen in the appropriate ELISA system and the final data was expressed as ng/ml.

Quantitation of the total accumulated complement component levels in each culture supernatant were determined by using the equation below and the final data was expressed as ng/ml:

$$Y_n = X_n + \sum_{(j=1)}^{\frac{n-1}{2}} \frac{X_{(2j-1)}}{2}$$

n = Days in culture, i.e. day 1, 3, 5, 7, 9 or 11.

X_n = Absolute complement protein detected in a culture supernatant obtained on day n .

Y_n = Total accumulated complement protein produced by cells in culture from day 0 to day n .

All data is expressed as the mean \pm the standard error of the mean (SEM), and statistical significance of the data was determined using the unpaired Student's t

test (19). Stimulation index was calculated using the following general equation:

$$SI = \frac{\text{Experimental value}}{\text{Control value}}$$

SIR = stimulation index for radioactive amino acid incorporation,

SIC3 = stimulation index for antigenic C3,

SIC5 = stimulation index for antigenic C5, and

SIC5f = stimulation index for functional C5.

RESULTS

Effect of Fetal Bovine Serum on Complement Component Production by A549 Pneumocytes

To begin our studies on complement component production by type II pneumocytes we first determined the effects of different lots of fetal bovine serum (FBS) on C3 and C5 production by A549 pneumocytes in vitro. Initially, we cultured A549 pneumocytes in two different lots of FBS for 11 days and analyzed culture supernatants obtained on days 1, 3, 5, 7, 9 and 11 for antigenic C3 and C5 levels by ELISA. Lot No. 2 of FBS stimulated a significantly greater rate of antigenic C3 production when compared to the effect of Lot No. 1, i.e. 335 ng C3/ml/24 hr versus 135 ng C3 ml/24 hr. Matched control cultures of A549 pneumocytes cultured in serum free medium produced antigenic C3 at similar rates (62 ng C3/ml/24/hr [Lot No. 1 studies] versus 70 ng C3/ml/24 hr [Lot No. 2 studies]). Additional studies with a third lot of FBS revealed similar effects on C3 production by A549 cells as described for Lot No. 2 (data not shown). Similar FBS effects were seen when these same A549 pneumocyte culture supernatants were analyzed for C5 production, i.e., Lot No. 2 stimulated antigenic C5 production at a rate of 320 ng C5/ml/24 hr in comparison to the rate of 245 ng C5/ml/24 hr determined for Lot No. 1. Once again control cultures of A549 pneumocytes cultured in serum free medium produced antigenic C5 at similar rates (148 ng C5/ml/24 hr versus 153 ng C5/ml/24 hr). It is important to note, although there were variations in the absolute amount of C3 and C5 produced by A549 pneumocytes cultured in different lots of FBS, the absolute amounts of C3 and C5 produced by A549 pneumocytes cultured in any individual lot of FBS remained

consistent from experiment to experiment. Additionally, when experimental results were expressed as a stimulation index the modulation of complement component production in control cultures as well as those exposed to silica particles was constant, with less than 10% variation within any experimental condition, regardless of the lot of FBS used (see below). Additionally, baseline C3 and C5 production by the A549 cells in serum free medium was similar in all the studies.

Toxicity of Silica Particles on A549 Pneumocytes

To begin our studies on silica induced modulation of complement component production by A549 pneumocytes, we examined the effects of silica on the viability of A549 cells in vitro by culturing these cells in the presence or absence of silica or FBS. Morphologically, A549 pneumocytes cultured in the presence of fetal bovine serum (FBS) with or without silica particles (1 mg/10⁷ cells/flask) remained a tightly packed epithelial cell monolayer. A549 cells cultured in the absence of FBS became spindle shaped and irregular (Figure 1A), and these effects, including cellular retraction, were more enhanced in the presence of silica particles (Figure 1B). Although the changes in A549 pneumocyte morphology detected in serum free cultures may likely be a result of subtle cell injury due to the lack of FBS as well as silica exposure, the dose of silica particles (1 mg/10⁷ cells/flask) used was not lethal, as A549 pneumocytes remained intact and attached to the tissue culture plastic substratum and excluded vital dyes. In addition, lactate dehydrogenase (LDH) levels in A549 cell culture supernatants were also measured, as an indicator of cell viability (18). The results of these studies demonstrated that A549 pneumocytes cultured in serum free medium with 1 mg silica particles/10⁷ cells for 7 days showed no significant release of total LDH (i.e., 1.0%) when compared to A549 pneumocytes cultured in serum free medium alone (1.3%). Increasing the silica concentration to 10 mg silica particles/10⁷ cells had significant toxic effects on A549 cells as reflected by a 70% increase in total LDH release, above that detected in non silica exposed cultures.

A549 pneumocyte integrity during exposure to silica particles was also assessed by both light and electron microscopy (Figure 2). Interestingly, silica particles were consistently associated with the cells in culture suggesting silica uptake by the A549 pneumocytes (see below). Light microscopy of wet mount

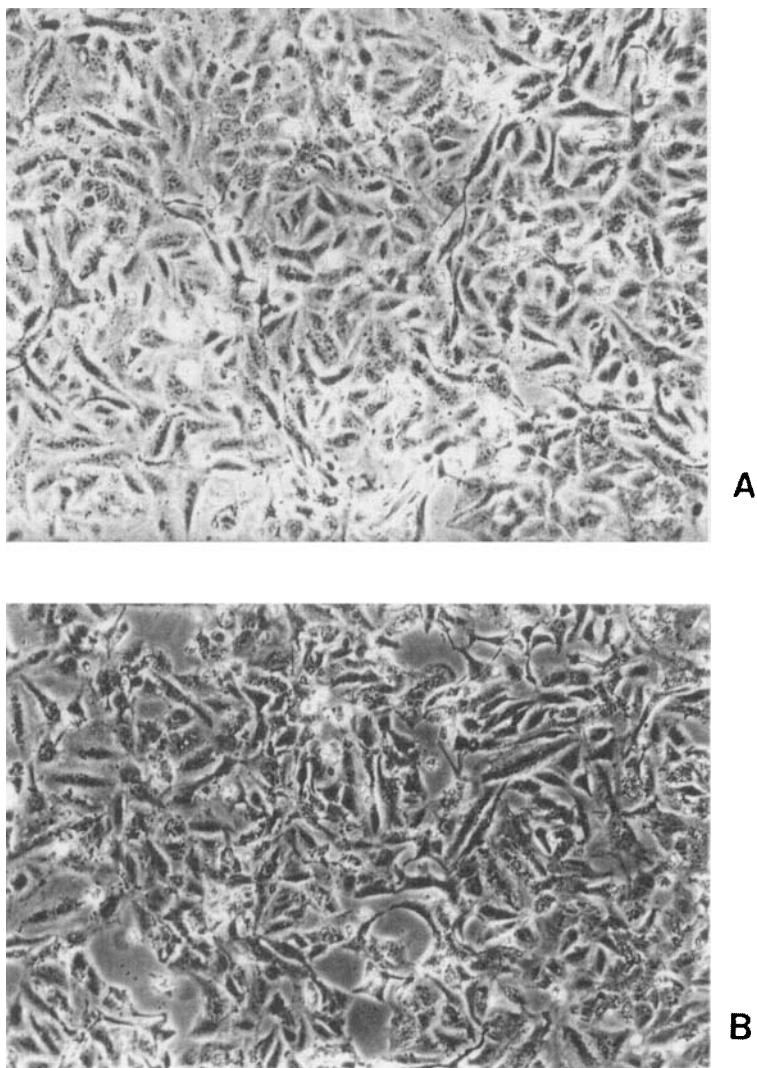


Figure 1: Phase contrast microscopic evaluation of silica particles and A549 pneumocytes.

Phase contrast micrographs of A549 pneumocytes after 5 days in culture in serum free medium, in the absence of silica particles (Figure 1A) or in the presence of silica particles (1 mg/10⁷ cells) (Figure 1B) x 100.

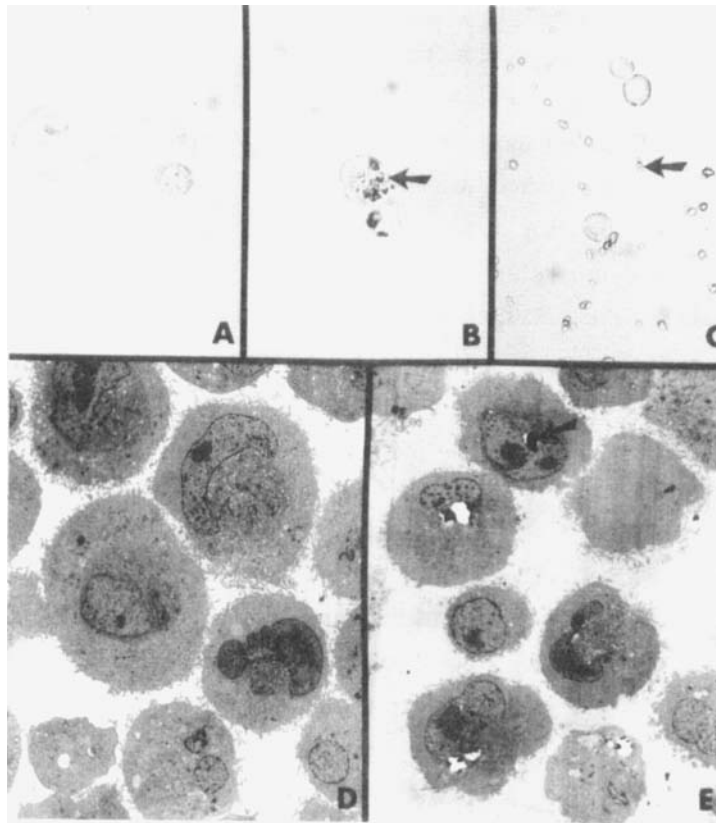


Figure 2. Light and electron microscopic evaluation of silica particle interaction with A549 pneumocytes.

Interactions of silica particles with adherent (cultured) and nonadherent (suspension) A549 pneumocytes were evaluated by light microscopy. Adherent A549 pneumocytes cultured in the presence of silica ($1 \text{ mg}/10^7$ cells) displayed normal cell morphology and consistent internalization of numerous silica particles (See arrow in Panel B) when compared to A549 cells cultured in the absence of silica (Panel A) or suspension cultures of A549 pneumocytes exposed to silica (See arrow in Panel C). Electron microscopic evaluation of A549 pneumocytes cultured in the presence of silica demonstrated that although the A549 cells internalized silica particles (See arrow in Panel E), there was no evidence of cellular toxicity, nor any major ultrastructural changes (e.g., normal lamellar bodies and cell surface microvilli) when compared to A549 pneumocytes cultured in the absence of silica (Panel D).

preparations of A549 pneumocytes after exposure to silica particles (1 mg/10⁷ cells/flask) for 5 days demonstrated that A549 cells internalized multiple silica particles and remained healthy (Figure 2, Panel B), and the interactions between silica particles and A549 pneumocytes were specific and not an artifact of sample preparation for microscopy (Figure 2, Panel C), since exposure of suspensions of A549 cells with silica for short time periods did not result in internalization of the silica (Figure 2). Our electron microscopy studies also showed A549 pneumocytes cultures exposed to silica particles phagocytized and internalized many silica particles per cell during culture *in vitro*. These *in vitro* observations are consistent with previous literature citations that have described type II pneumocytes *in situ* to have phagocytic capabilities (20-23). The non-toxic effects of the 1 mg dose of silica was supported by electron microscopic evaluation, i.e., A549 pneumocytes cultured in the presence of silica particles (1 mg/10⁷ cells/flask) for 5 days appeared to be in excellent morphological condition, that is displayed normal cytoplasmic organelles, including numerous cytoplasmic multilamellar bodies, lipid filled vacuoles and cell surface microvilli (Figure 2, Panel E). Evidence of cellular dysfunction involving nuclear disorganization, cytoplasmic vacuolization or loss and disorganization of lamellar bodies was not detected.

Silica and C3 Production by A549 Pneumocytes

To begin our studies on the effects of silica on C3 production by A549 pneumocytes we initially analyzed culture supernatants obtained on days 1, 3, 5, 7, 9 and 11 post silica exposure for antigenic C3 levels. As shown in Figure 3, A549 pneumocytes cultured in serum free medium plus a non-lethal dose of silica produced only slightly greater total accumulated antigenic C3 levels in comparison to A549 cells cultured in serum free medium without silica particles. Analysis of the data from a representative experiment (Figure 3) demonstrated that generally after 3 days in culture, A549 pneumocytes cultured in serum free medium produced C3 at a rate of 78.8 ± 29.4 ng C3/ml/24 hr (mean \pm SEM), which is similar to the rate of 88.5 ± 41.5 ng C3/ml/24 hr determined for A549 cells cultured in serum free medium plus silica particles. In contrast, A549 pneumocytes maintained in medium containing FBS and silica particles produced significantly lower absolute amounts of antigenic C3 which resulted in depressed total accumulated levels

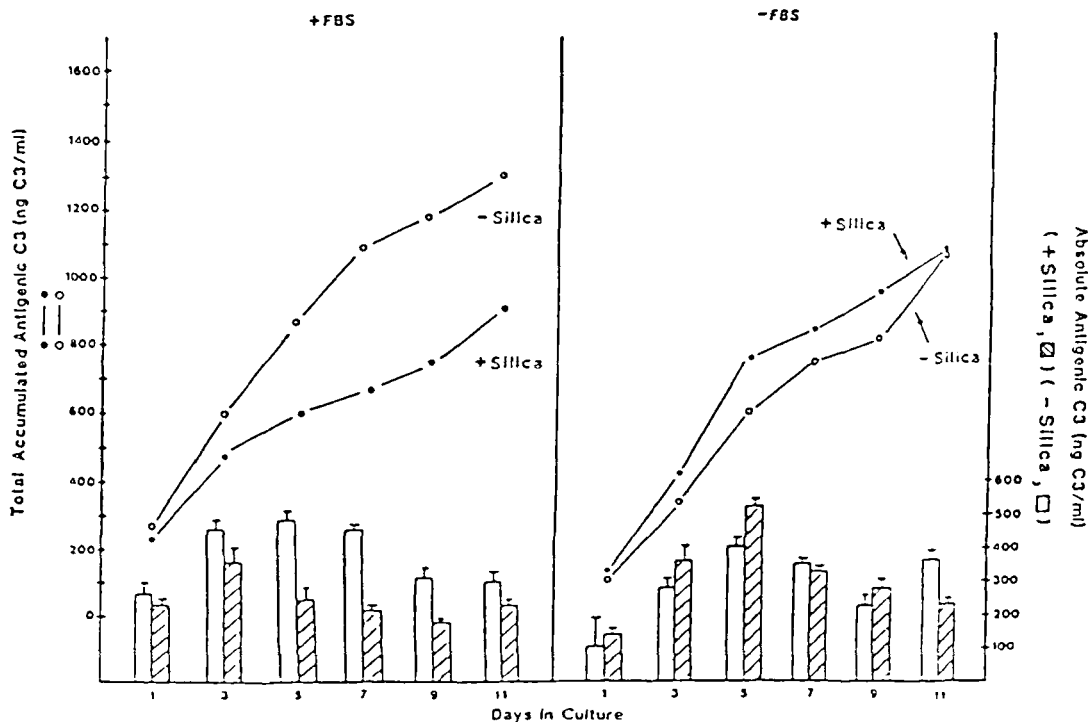


Figure 3. Effect of silica on C3 production by A549 pneumocytes.

Figure 3 demonstrates the kinetics of C3 production by A549 pneumocytes under various experimental conditions. The open bars represent absolute antigenic C3 levels (ng/ml) detected in culture supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS for 11 days. The hatched bars represent absolute antigenic C3 levels (ng/ml) in supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS, and 1 mg silica particles for 11 days. The line graphs represent the total accumulated antigenic C3 (ng/ml) produced by A549 pneumocytes under the conditions described above.

during 11 days in culture, in comparison to A549 cells cultured without silica particles. Analysis of the data in Figure 3 showed that after 3 days in culture, A549 pneumocytes cultured in FBS containing medium plus silica generally produced C3 at a rate of 42.0 ± 4.7 ng C3/ml/24 hr which is significantly slower than the rate calculated for A549 cells cultured in FBS containing medium alone, 94.0 ± 23.5 ng C3/ml/24 hr ($p < 0.01$). To eliminate the possibility that depressed levels of antigenic C3 seen in the experiments described above were not simply a result of

non-specific sticking of C3 molecules to silica particles, we demonstrated that neither C3 or C5 would adhere to silica (see material and methods section). When data from 5 independent experiments were analyzed for the stimulation index for C3 production (Figure 4) it can be seen that: 1) A549 pneumocytes cultured in serum free medium plus silica particles had a maximum $SI_{C3} = 1.12 \pm 0.06$ indicating negligible effects on C3 production, and 2) A549 cells cultured in FBS containing medium plus silica particles showed significant suppression of C3 production, e.g., by day 11 the $SI_{C3} = 0.58 \pm 0.02$ (Figure 4).

Silica and C5 Production by A549 Pneumocytes

Given the importance of C3 and C5 during an inflammatory reaction and the observation that silica particles could suppress A549 pneumocyte production of C3, we next investigated the ability of silica to modulate C5 production. For these studies, these same A549 pneumocyte culture supernatants were analyzed for effects of silica particles on antigenic C5 levels using an ELISA technique. Since the ELISA only measured antigenic C5 levels, and could not distinguish functional C5 proteins from non-functional C5 proteins, we also examined the effects of silica particles on functional C5 protein levels using a hemolytic titration assay. In general, A549 cells cultured in serum free medium plus a non-lethal dose of silica ($1 \text{ mg}/10^7 \text{ cells/flask}$) produced similar levels of absolute and total accumulated antigenic C5 when compared to A549 cells cultured in serum free medium without silica particles (Figure 5). Specifically, A549 pneumocytes cultured in serum free conditions produced antigenic C5 at a rate almost identical to that determined for A549 cells exposed to silica particles, i.e., $148.5 \pm 11.8 \text{ ng C5/ml/24 hr}$ versus $148.3 \pm 37.3 \text{ ng C5/ml/24 hr}$. Analysis of functional C5 levels in these same A549 pneumocyte culture supernatants demonstrated that exposure to silica also had no dramatic effect on functional C5 production (Figure 6) and that the rate of functional C5 production was $122.6 \pm 11.7 \text{ ng C5/ml/24 hr}$ for A549 cells in serum free medium, and $97.8 \pm 10.1 \text{ ng C5/ml/24 hr}$ for A549 cells exposed to silica particles. In contrast, A549 pneumocytes cultured in medium supplemented with FBS and silica particles produced significantly lower levels of both absolute and total accumulated antigenic C5 and functional C5 when compared to A549 cells cultured without silica particle insult (Figure 5 and Figure 6). Analysis of data in Figure 5

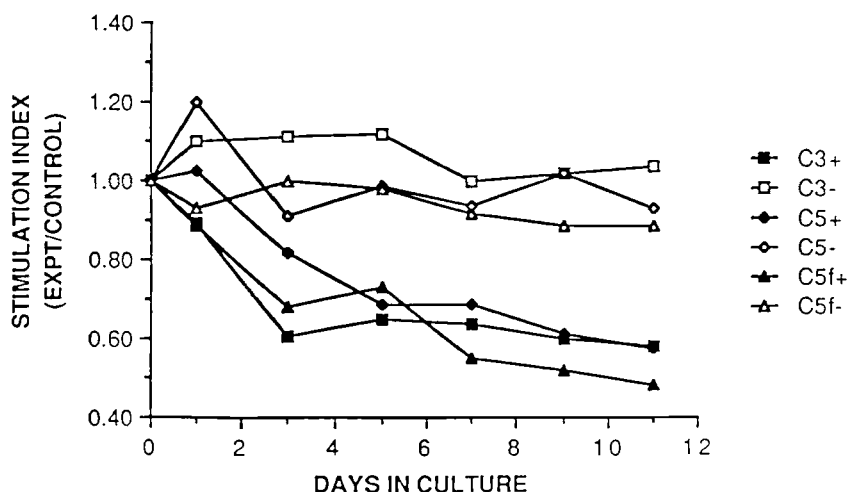


Figure 4. Effect of silica on C3 and C5 production by A549 pneumocytes.

The open symbols represent the mean stimulation index (SI) calculated for culture supernatants obtained from A549 pneumocyte cultures maintained in medium without 15% FBS plus 1 mg silica particles for 11 days. The line graphs with the open symbols represent SI for antigenic C3 ($n=7$), antigenic C5 ($n=7$), and functional C5 ($n=7$). The closed symbols represent the mean SI calculated for culture supernatants obtained from A549 pneumocyte cultures maintained in medium with 15% FBS plus 1 mg silica particles for 11 days. The line graphs with the closed symbols represent SI for antigenic C3 ($n=5$), antigenic C5 ($n=4$), and functional C5 ($n=4$). The SEM was less than 10% for all data presented.

showed that after 5 days in culture, A549 pneumocytes cultured in FBS containing medium plus silica produced antigenic C5 at a rate of 129.3 ± 11.3 ng C5/ml/24 hr, which is significantly slower than the 273.0 ± 70.2 ng C5/ml/24 hr determined for A549 cells cultured without silica particles ($p < 0.02$). As shown in Figure 6, functional C5 production was also significantly suppressed when A549 cells were exposed to silica particles for 11 days, i.e., the rate of functional C5 production was 105.3 ± 30.0 ng C5/ml/24 hr versus 212.8 ± 35.4 ng C5/ml/24 hr ($p < 0.05$). These patterns of modulation by a non-lethal dose of silica on C5 production by A549 pneumocytes were documented in several independent experiments as shown in Figure 4. Specifically, A549 pneumocytes cultured in serum free medium plus silica particles had a stimulation index for antigenic C5 production (SI_{C5} : mean \pm SEM) of $SI_{C5} = 1.00 \pm 0.04$ ($n=7$), and a stimulation index for functional C5

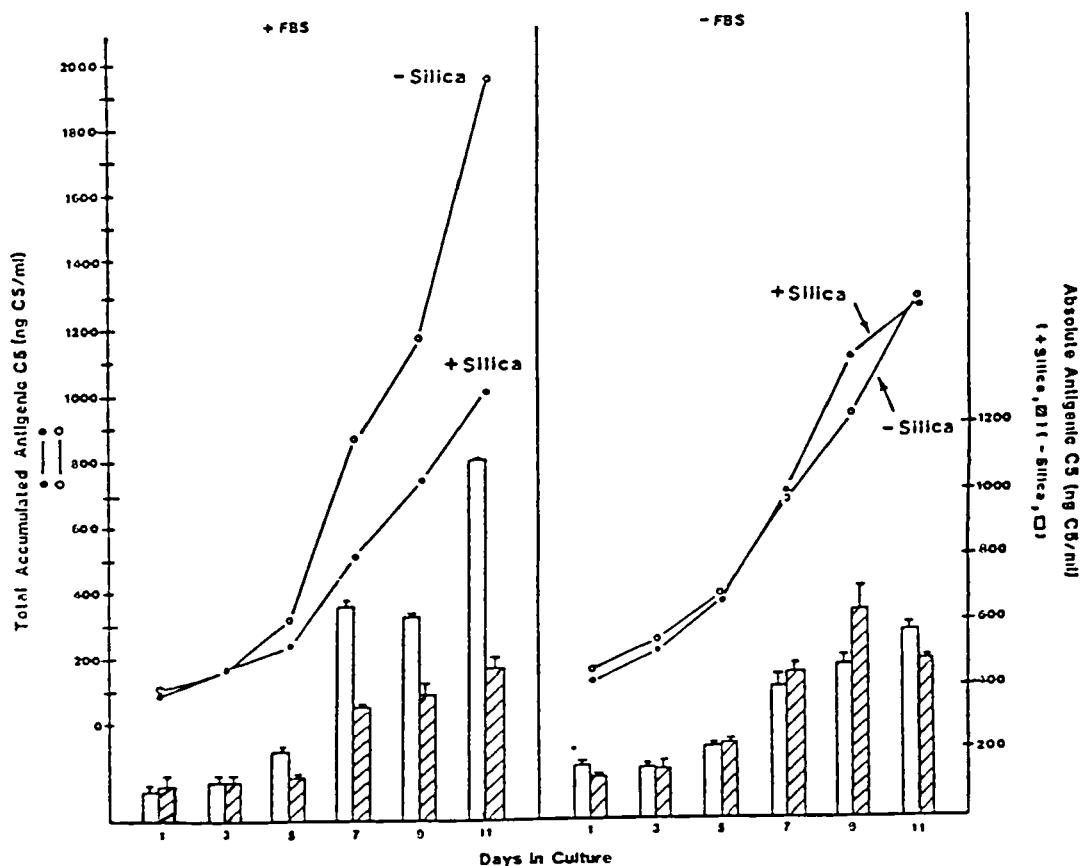


Figure 5. Effect of silica on C5 production by A549 pneumocytes.

The open bars represent absolute antigenic C5 levels (ng/ml) detected in culture supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS for 11 days. The hatched bars represent absolute antigenic C5 levels (ng/ml) in supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS, and 1 mg silica particles for 11 days. The line graphs represent the total accumulated antigenic C5 (ng/ml) produced by A549 pneumocytes under the conditions described above.

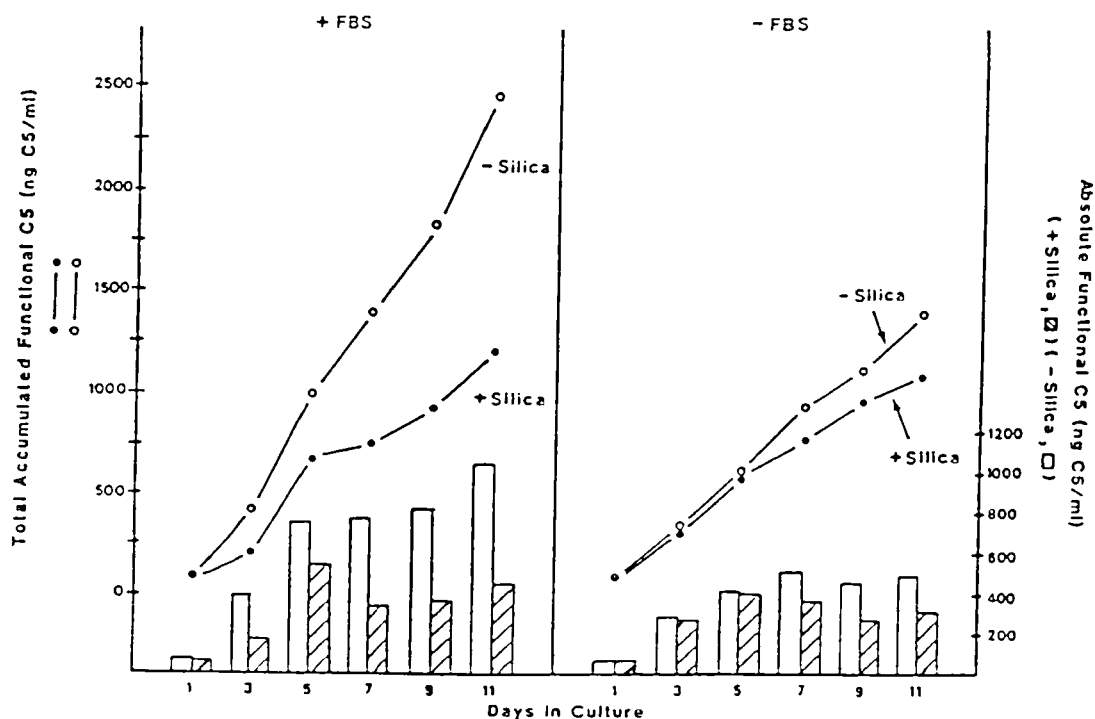


Figure 6. Effect of silica on functional C5 production by A549 pneumocytes.

The open bars represent absolute functional C5 levels (ng/ml) detected in culture supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS for 11 days. The hatched bars represent absolute functional C5 levels (ng/ml) in supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS, and 1 mg silica particles for 11 days. The line graphs represent the total accumulated functional C5 (ng/ml) produced by A549 pneumocytes under the conditions described above.

production (SI_{C5f}: mean \pm SEM) of SI_{C5f} = 0.94 ± 0.02 (n=7) during 11 days in culture. In addition, analysis of culture supernatants from at least 4 independent experiments of A549 pneumocytes cultured in medium supplemented with FBS and silica demonstrated suppression of C5 production by day 3 in culture, with maximum suppression detected on day 11 for both antigenic C5 and functional C5, i.e., SI_{C5} = 0.58 ± 0.05 and SI_{C5f} = 0.48 ± 0.00 , respectively (Figure 4).

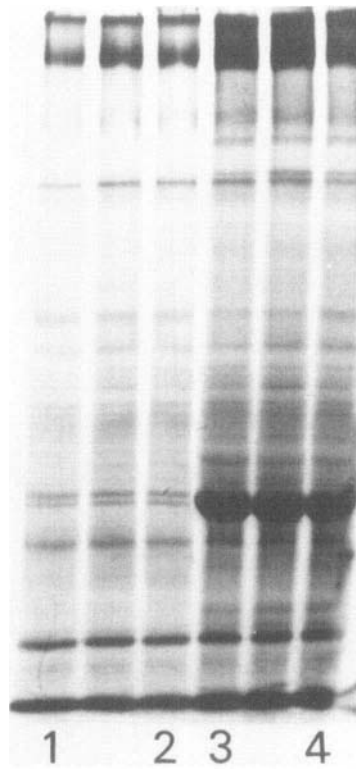


Figure 7. Effect of silica particles on total protein synthesis and secretion by A549 pneumocytes.

Figure 7 is an autoradiograph of a SDS-PAGE (7.5%) of culture supernatants obtained from A549 pneumocyte cultures that were metabolically labeled for 3 days with ^{35}S -methionine. Lane 1, serum free A549 cell culture supernatant; Lane 2, serum free A549 cell culture supernatant with silica particles (20.8 μg silica/48 well); Lane 3, 15% FBS A549 cell culture supernatant; and Lane 4, 15% FBS A549 cell culture supernatant with silica particles (20.8 μg silica/48 well).

Silica and Protein Synthesis by A549 Pneumocytes

Since silica induced suppression of complement component production by A549 pneumocytes may have been a result of non-specific suppression of total protein synthesis (a response due to cell injury) or specific suppression of C3 and C5 production (i.e., specific effects on gene expression and/or secretion) we characterized the effects of silica on general protein synthesis. A549 cells were

cultured in methionine free medium supplemented with ^{35}S -methionine and the effects of silica exposure on total protein synthesis were quantitated as percent TCA precipitable radioactivity, i.e., radiolabelled proteins. Data from these experiments demonstrated that there was no significant difference in ^{35}S -methionine incorporation into total protein, when A549 cells were cultured with or without a non-lethal dose of silica. The stimulation index for radioactive amino acid incorporation (SIR : Silica/control mean \pm SEM) demonstrated that silica had no effect on total protein synthesis by A549 pneumocytes, i.e., $\text{SIR} = 1.05 \pm 0.02$ for serum free cultures ($n=2$), and a $\text{SIR} = 1.20 \pm 0.01$ for FBS cultures ($n=2$). Although silica particles did not effect total protein synthesis by A549 pneumocytes it may have altered the synthesis of specific proteins thus we analyzed ^{35}S -methionine labelled culture supernatants by 7.5% SDS-PAGE followed by autoradiography to examine the qualitative differences in protein synthesis. Figure 7 shows an autoradiograph of a representative PAGE which demonstrates that there are no apparent qualitative differences in the array of proteins synthesized and secreted by A549 pneumocytes in the presence or absence of silica particles. Not unexpectedly, culturing A549 pneumocytes with or without silica in the presence of cycloheximide (1 mg/ml) caused inhibition of not only C3 and C5 production, but total protein synthesis which was reversed by the removal of the cycloheximide (data not shown). In summary, these data eliminate the possibility that silica particle modulation of C3 and C5 production is a result of generalized effects of silica on total protein synthesis by A549 pneumocytes, and suggest that the suppression of C3 and C5 production during exposure to silica particles may be a reflection of specific effects on either gene expression (pre-translational level) or secretion (post-translational level) of the third and fifth complement components.

Effect of Silica on Complement Component Production by WI-38 Lung Fibroblasts

To determine whether silica particles could modulate complement component production by other isolated human lung cells, we next evaluated the effects of a non-lethal dose (i.e., $1 \text{ mg}/10^7 \text{ cells/flask}$) of silica on WI-38 fibroblast production of C3 and C5. Confluent cultures of WI-38 fibroblasts were maintained for 11 days in the presence or absence of FBS, with or without silica particles. Under microscopic examination, WI-38 fibroblasts cultured in serum free

or FBS containing medium formed monolayers of numerous swirls of elongated cells oriented in parallel to each other. The addition of a non-lethal dose of silica (1 mg/flask) had no effect on fibroblast cell morphology. At selected times during the 11 day culture period, culture supernatants were obtained, and analyzed for antigenic C3 and antigenic C5 levels. The results showed that the presence of silica particles in WI-38 fibroblast cultures, maintained in serum free medium, did not modulate antigenic C3 or antigenic C5 levels when compared to WI-38 fibroblasts maintained in the absence of silica, i.e., the $SI_{C3} = 1.09 \pm 0.07$ (n=7) and the $SI_{C5} = 1.00 \pm 0.03$ (n=12). Similar data was obtained for WI-38 fibroblasts cultured in medium supplemented with FBS. Therefore, it appears that not all human lung cells are susceptible to the modulating effects of a non-lethal dose of silica particles on their capability to produce complement components *in vitro*.

DISCUSSION

Although previous studies have focused on various aspects of complement component production by inflammatory cells (i.e., monocyte/macrophage), very little is known about the capability of normal or injured tissue cells to produce complement components *in vivo* or *in vitro*. Thus to fill this important gap area, our laboratory has recently studied complement production by lung type II pneumocytes (A549) and lung fibroblasts (WI-38) as prototypes of tissue specific cells. Using ^{35}S -methionine incorporation and immunoprecipitation of specific complement components, these investigations have demonstrated the capability of type II pneumocytes to synthesize and secrete numerous complement components and regulatory proteins including C1r, C1s, C4, C3, C5, C6, C7, C8, C9, Factor B, Factor H, Factor I and C1s inactivator *in vitro* (10). In comparison, lung fibroblasts were shown to synthesize C1s, C4, C3, C5, C6, C8 and C9. In summary, these studies revealed four important findings: 1) *in vitro*, A549 pneumocytes and WI-38 fibroblasts can synthesize and secrete a wide variety of early and terminal complement components, 2) lung cells synthesize and secrete a number of complement components (e.g., C6, C7, C8, C9 and C1s inactivator), not described to be produced by the monocyte/macrophage, 3) type II pneumocytes produce significantly greater amounts of C3 (20 fold increase), as quantitated by an enzyme-linked immunospecific assay (ELISA), than that reported for the monocyte (24),

and 4) using ^{35}S -methionine incorporation and immunoprecipitation techniques, the C3 and C5 produced by the type II pneumocytes is physiochemically identical to serum C3 and C5. These data clearly indicate that on a per cell basis, the type II pneumocyte likely represents a major source of local complement component production within the lung. This data assumes additional importance in light of the fact that within the normal lung alveoli, tissue epithelial cells are far more numerous than alveolar macrophages, and that the number of type II pneumocytes markedly increases as a result of lung injury such as silica inhalation (11,25-29). Clearly, agents or factors which enhance or suppress production of complement components by these tissue cells would likely have a significant impact on the resolution of lung diseases.

Silicosis has long been recognized as a major health hazard associated with a wide variety of occupations (30). Generally, silicosis involves the inhalation of silica particles penetrating deep into lung tissue, which is followed by the uptake of the particles by lung cells including macrophages and epithelial cells (30,31). Once ingested, silica eventually leads to cell destruction, increased accumulation of cells, and ultimately, production of collagen and hyaline membrane formation within the lung (30). In addition to the pathologic effects of silica on the lung, a variety of studies have demonstrated that silica suppresses immune function. For example, silica is known to decrease antibody production *in vivo* as well as macrophage and lymphocyte function *in vitro* (32,33). Additional studies have demonstrated the presence of complement-dependent chemotactic factors in the lavage fluid of patients with silicosis, thus a role for the complement system in the etiology of silicosis has been suggested (31). Since silica has been directly demonstrated to induce pulmonary inflammation and induce immunosuppression (30-34), we examined the effects of silica on complement production by type II pneumocytes and lung fibroblasts. In our present studies using sensitive ELISAs, we demonstrated that silica particles had the capability to suppress both C3 and C5 production by type II pneumocytes by 40-50 percent without alteration of total protein synthesis as demonstrated by ^{35}S -methionine incorporation studies. These data suggest that suppression of both C3 and C5 production was a specific response rather than a generalized response of the type II pneumocyte to silica. Furthermore, the C3 and C5 levels produced by A549 pneumocytes cultured in the presence of FBS and absence of silica particles were significantly higher than the C3 and C5 levels produced by A549 cells cultured in 1) the presence of FBS and

silica, or 2) cells cultured in serum free conditions in the presence or absence of silica. These results suggest that complement production by A549 pneumocytes is responsive to not only silica, but also to culture conditions, i.e., a nutrient-deprived environment (such as in serum free culture conditions), and factors within serum (cytokines, growth factors, hormones, etc.). Interestingly, silica had no effect on C3 or C5 production by WI-38 fibroblasts. Thus, these studies not only demonstrate the capability of silica, which has been implicated in pulmonary inflammation, to regulate complement production by type II pneumocytes, but that this regulation may be unique to the type II cell since it does not occur in the lung fibroblast. These observations suggest that during pulmonary disease such as silicosis, resident lung cells (such as type II pneumocytes) may have a direct impact on pulmonary host defense and inflammation by directly altering tissue levels of important complement components such as C3 and C5.

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FOOTNOTES

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